

# Disseminated tumor cells in breast cancer: detection, characterization and clinical relevance

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Hematogenous distant metastasis is the leading cause of cancer-related death in breast cancer and other solid tumors. By applying sensitive immunocytochemical or molecular assays, disseminated tumor cells (DTCs) in bone marrow can be detected in 20–40% of breast cancer patients without any clinical or even histopathological signs of metastasis. The detection of DTCs provides prognostic information and might help to identify patients who need adjuvant therapy, and to monitor the efficacy of adjuvant therapy. Within the last few years, various efforts have led to an increased sensitivity in the detection of DTC. This review will summarize the most important methods for DTC detection in bone marrow and for circulating tumor cells in the blood of breast cancer patients, the clinical relevance of DTCs and, finally, provide an outlook on clinical implications.

Metastasis is the leading cause of death in breast cancer. As early as 1889, Paget described an association between breast cancer and the development of bone metastases [1]. Over 40 years later, Rohr and Hegglin proposed that bone marrow (BM) biopsies could be used to detect metastatic cells in BM with conventional histological hematoxylin and eosin (H&E) staining procedures [2]. Introduction of Quensel's nucleus-nucleoli relation [3] to the histological characteristics further facilitated the discrimination of metastatic cancer cells from the surrounding BM cells. However, Bauer stated that not all morphological criteria are sufficient to distinguish cancer cells from normal cells [4]. In 1954, Schreiber described the detection of single disseminated tumor cells in BM smears obtained from four breast cancer patients without metastatic disease [5]. However, only a small number of groups focused on micrometastasis at this time [6]. A great development in the research field of micrometastasis was the detection of disseminated tumor cells (DTCs) with immunocytochemical staining approaches using antibodies against epithelial specific markers (e.g., epithelial membrane antigen [EMA] and cytokeratins [CKs]) that are not expressed on surrounding mesenchymal BM cells [7]. However, all these studies were small, uni-center reports without a clear definition of the terminology for micrometastases or DTCs in contrast to solid metastases. Since these pioneering publications, 288 articles were published, focusing on the detection and prognostic relevance of micrometastases in the BM of breast cancer patients (National Center for Biotechnology Information [NCBI], PubMed database). This review focuses on the detection, clinical relevance and characterization of DTCs in the BM of breast cancer patients.

## Detection of DTCs

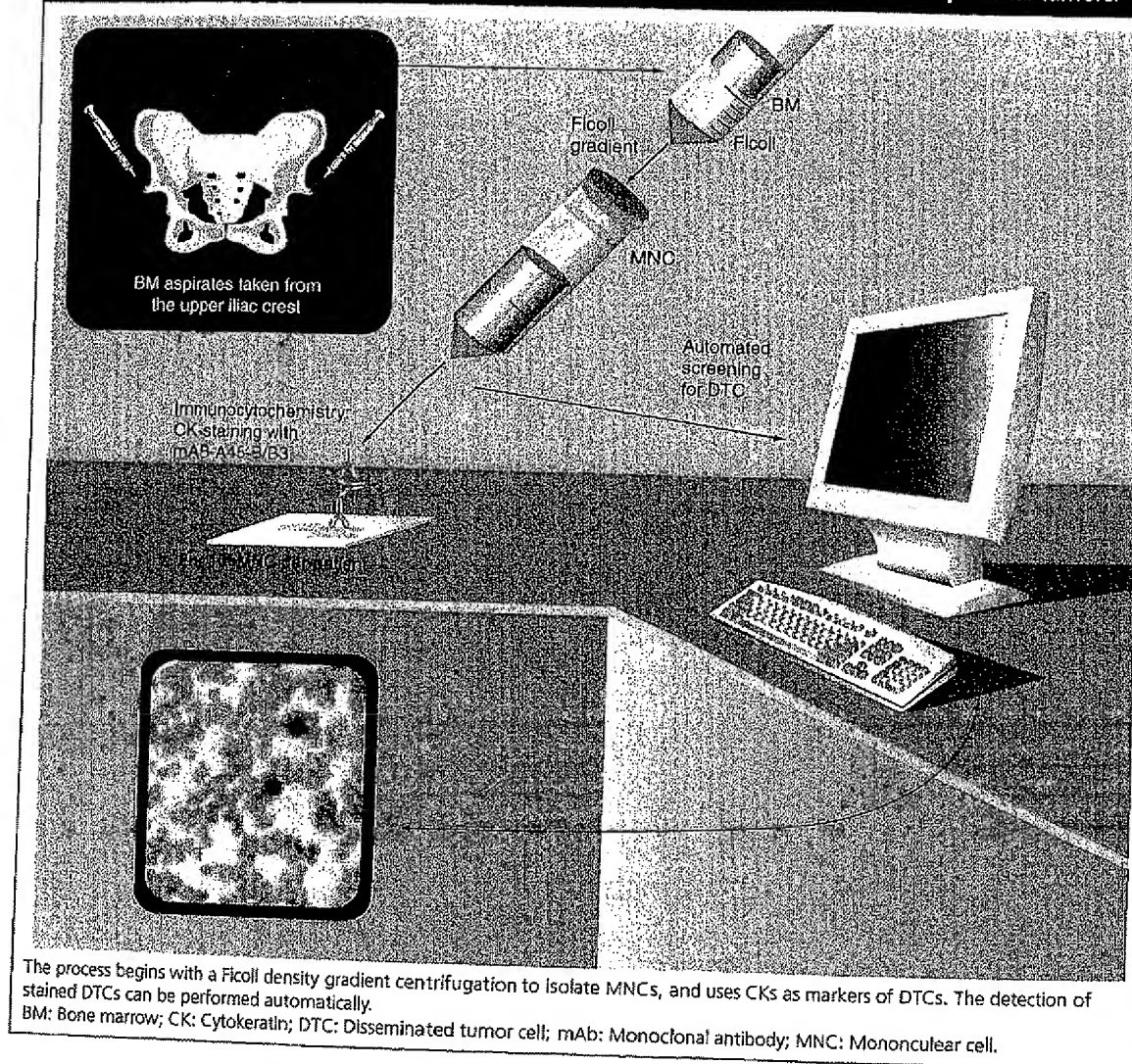
### Immunocytochemical staining

Over the past decades, many different assays have been developed to detect DTCs in breast cancer and other types of carcinomas. One major approach to identify DTCs from BM after aspiration includes density gradient centrifugation with subsequent immunocytochemical staining using monoclonal antibodies against epithelial or tumor-associated antigens (Figure 1). A total of 24 different mono- and polyclonal antibodies or antibody cocktails directed against different antigens were used for immunocytochemical identification of DTCs in BM. Many groups have used antibodies against EMA, directed against an epithelial cell-surface antigen [8], TAG12, a tumor-associated glycoprotein [9] and CKs, the structural proteins of the epithelial cytoskeleton [10,11]. To date, CKs have become the most widely accepted protein markers in such immunocytochemical assays. A combination of several antibodies to various CK antigens, or an antibody against a common epitope present on various CK proteins (e.g., A45B/B3 directed against CK8, 18 or 19, among others), appear to be superior to monospecific antibodies directed against a single CK protein (e.g., CK2 against CK18) [11–13], owing to the considerable antigenic heterogeneity of solid tumor cells. Although low level illegitimate expression of CK mRNA has been observed in normal BM and lymph node cells [14,15], it obviously never leads to sufficient amounts of CK proteins to be detected by immunocytochemical methods. With this approach, one single DTC can be detected in the background of millions of BM cells.

Keywords: bone marrow, cancer, cytokeratin, immunocytochemistry, micrometastasis, PCR

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Figure 1. Immunocytochemical detection of disseminated tumor cells in patients with epithelial tumors.



However, different staining techniques can result in specificity variations. Hematopoietic cells can be directly reactive to alkaline phosphatase [16] or produce endogenous peroxidase [17], consequently resulting in false-positive staining in alkaline phosphatase-based or peroxidase-based methods, if these enzymes were not fully blocked. This demonstrates that the immuno-cytochemical technique is dependent on the reaction parameters. Several international organizations have recognized the need for standardization of the immunocytochemical assay and for its

evaluation in prospective studies [18,19]. Furthermore, the development of an improved immunocytochemical detection platform is also part of the objectives of the newly established European consortium disseminated malignancies (DISMAL), funded by the European Commission and coordinated by K Pantel.

The use of new automated devices for the microscopic screening of immunostained slides may help to read slides more rapidly and to increase reproducibility of the read-out [20-25]. Another way to improve current detection assays for single tumor cells is to develop better

tumor-cell enrichment procedures using improved density gradients [26] and antibody-coupled magnetic particles [20,27,28]. At present, it is unclear whether these new enrichment techniques provide more clinically relevant information than the standard density gradient procedure used to isolate the mononuclear cell fraction.

Clinically relevant data on the highest level of evidence (LOE-I) demonstrates that DTC detection by immunohistochemistry provides clinically relevant information [11]. Although quantitative PCR and cytogenetic methods are also interesting approaches, the illegitimate expression of the marker transcript and the substantial genetic heterogeneity of solid tumors have so far posed great problems to the widespread use of these molecular technologies. These problems need to be overcome and may be the reason why, to date, LOE-I data are only available for immunocytochemical assays. Immunocytochemistry with anti-CK antibodies certainly has some drawbacks (e.g., subjective read-out and nonspecific staining of plasma cells), but has been used by many groups on thousands of patients for DTC detection in BM, blood and lymph nodes over the past 10 years and is now broadly accepted as a kind of standard [29]. Future developments might, however, lead to the replacement of this technology.

#### PCR approach

A widely used alternative to immunocytochemical assays for the detection of DTCs is molecular detection procedures. In principle, the nucleic acid in a sample can be amplified by PCR, so that very small numbers of tumor cells can be detected in a heterogeneous population of cells. However, the tumor cells must have changes in their DNA or mRNA expression pattern that distinguishes them from the surrounding hematopoietic cells. At the DNA level, breast carcinomas are genetically quite heterogeneous, therefore there is no universally applicable DNA marker available. Consequently, the main approach to developing molecular diagnostic assays for breast carcinomas has focused on RNA markers. As primary tumors might continuously evolve genetically over time in response to host pressures and treatment, a multimarker approach with a panel of tumor-specific mRNA markers may improve the sensitivity for the detection of DTCs over single marker assays [30,31].

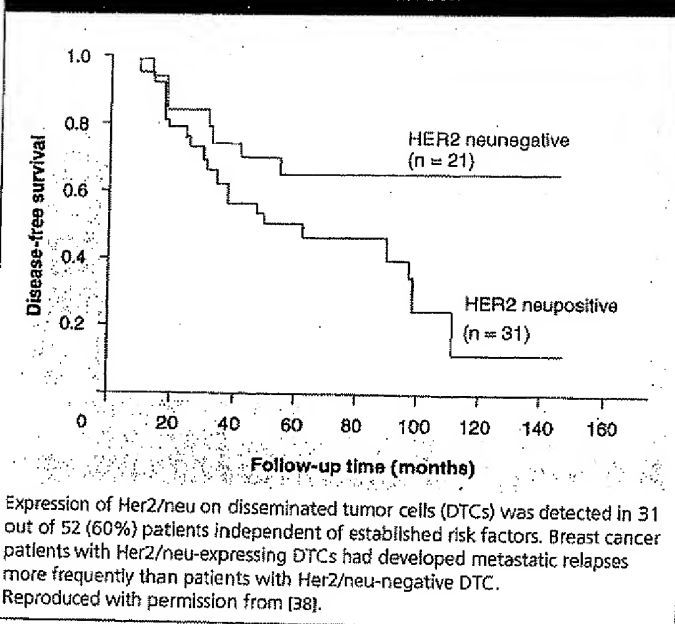
To date, many transcripts have been evaluated as 'tumor-specific' markers, such as CK18, -19, -20, Mucin-1 and carcinoembryonic antigen [32]. However, many of these transcripts can also be identified by reverse transcriptase (RT)-PCR in normal BM, blood and lymph node tissue [14,15,33]. Pre-analytical depletion of the disturbing normal cell fraction (e.g., granulocytes that express CK20) or quantitative RT-PCR determinations might solve this severe problem. However, Dandachi and colleagues demonstrated that pre-analytic positive selection of tumor cells using anti-HEA monoclonal antibody-coated magnetic beads and subsequent real-time RT-PCR for CK20 still resulted in a high percentage of false-positive signals in normal controls [34]. As CK20 is a tissue specific marker for epithelial cells of the gastrointestinal tract, patients with chronic inflammatory diseases, such as Crohn's disease or ulcerative colitis, also have a high rate of positivity for CK20 signal in the peripheral blood. Whether this signal originates from circulating gastrointestinal cells in these patients, or from higher levels of granulocytes in the circulation because they have not been adequately eliminated by positive selection, is not known.

#### Molecular & functional characterization of DTCs

DTCs in BM of breast cancer patients have been characterized with immunological double staining to identify biological features that might favor early dissemination. Fluorescence *in situ* hybridization (FISH) for CK mRNA expression is prone to technical problems (e.g., RNA stability and illegitimate transcription of RNA) and prominent oncogenes, for example Her2/neu, are only amplified in approximately 20% of breast cancers. However, for further characterization of CK<sup>+</sup> cells, methods other than FISH are well suited to assess the genetic make-up of DTCs [35]. Multiple characterization approaches of DTCs in BM show a considerable phenotypic heterogeneity; in particular the Her2/neu proto-oncogene appears to define a very aggressive subset of DTCs with increased invasive capability [36] and has gained substantial importance as a biological target for systemic therapy in breast cancer [37].

It could be demonstrated that the presence of Her2/neu expressing DTCs are also associated with impaired prognosis (Figure 2) [38]. Furthermore, there is some evidence of a prognostic effect of HER2-positive circulating tumor cells (CTCs) in Stage I-III breast cancer [39]. Fox and colleagues

**Figure 2. Prognostic relevance of Her2/neu-expression disseminated tumor cells in bone marrow.**



evaluated another factor that might favor early dissemination by studying the association between the presence of DTCs in BM and tumor angiogenesis and vascular invasion [40]. Furthermore, most DTCs and CTCs do not express the proliferation antigen Ki-67 and may therefore be resistant to chemotherapy [41,42].

A detailed molecular description of DTCs in BM of breast cancer patients without clinical signs of overt metastases demonstrated that these cells are genetically heterogeneous [43] and lacked genomic aberrations observed in arbitrary selected areas of the primary tumors [44].

By applying gene expression analysis on primary breast tumors in relation to the presence or absence of DTCs in BM, we observed a specific gene signature in primary tumors of patients with DTCs in BM [45]. These findings challenge the traditional concept that tumor cells acquire their metastatic genotype and phenotype late during tumor development, but rather support the alternative concept that tumor cells acquire the genetic changes relevant to their metastatic capacity early in tumorigenesis [46], so that the metastatic potential of human tumors is encoded in the bulk of a primary tumor [46,47]. This concept could also explain the presence of DTCs in BM at early stages of breast cancer.

#### Clinical relevance of DTC detection in BM

Despite the progress made in recent years, the prognosis of patients with breast cancer is still limited by metastatic relapse even in small primary tumors, which again indicates an early tumor cell spread.

In 1983, Redding and colleagues published the first paper on the prognostic importance of DTCs in BM of breast cancer patients [48]. It was shown that the presence of DTCs in BM was detectable in 20–40% of breast cancer patients investigated [11]. It is noteworthy that a similar prevalence was found in all other carcinoma types and, until now, no report has demonstrated a solid tumor type without immunocytochemically detectable epithelial cells in BM. In fact, DTCs have also been found in the BM of patients with colon cancer that rarely metastasize to the bone [49]. Therefore, BM might be an important reservoir that allows DTC to adapt and disseminate into other organs.

Many studies have demonstrated a correlation between the presence of DTCs in BM and an impaired prognosis (Table 1). Nevertheless, there are also several studies that could not confirm BM as an independent prognostic indicator [50,51]. A previous meta-analysis including 20 older studies of nearly 2500 patients suggested that the detection of DTCs offer no additional prognostic information over the established prognostic factors [52]. However, these studies are associated with inherent problems, in that the detection methods, antibodies used and the number of cells analyzed was not according to procedures currently regarded as standards. Furthermore, the patient cohorts were small and the follow-up data relatively short. These deficiencies underline the requirement for standardized detection and quantification procedures. A recent pooled analysis of more than 4700 breast cancer patients with Stage I, II or III breast cancer without overt metastases from nine independent studies demonstrated that the presence of DTCs in BM was associated with; larger tumors, a higher histological grade, lymph node metastases and hormone receptor-negative tumors [11]. Subgroup analysis showed that BM micrometastasis was associated with worse outcomes at all risk levels, even among those with small tumors and without lymph node involvement, indicating prognostic relevance in all subgroups (Figure 3). In these large trials analyzed, most investigators used antibodies against CKs to detect DTCs in BM.

**Table 1. Prognostic relevance of disseminated tumor cells identified by immunocytochemistry in bone marrow of breast cancer patients without overt distant metastases (stage M0).**

Study	Marker (antibody)	Detection rate (%)	Prognostic value (no. of patients)	Ref.
Schlimok	CK18	18	DDFS (155)	[61]
Porro <i>et al.</i>	Glycolipid	17	None (159)	[62]
Cote <i>et al.</i>	Mucin/CK	37	DFS, OS (49)	[63]
Dier <i>et al.</i>	Mucin: TAG12 (2E11)	31	DFS*, OS* (727)	[64]
Molino <i>et al.</i>	Mucin	31	None (109)	[50]
Mansi <i>et al.</i>	Mucin: EMA (anti-EMA)	25	DFS, OS (350)	[65]
Braun <i>et al.</i>	CKs (A45B/B3)	36	DFS*, OS* (552)	[13]
Gebauer <i>et al.</i>	EMA, CKs (CAM 5.2)	42	DFS*, OS* (393)	[8]
Gerber <i>et al.</i>	CKs (5D3)	31	DFS*, OS* (484)	[10]
Wiedswang <i>et al.</i>	CKs (AE1/AE3)	13	DDFS, BCSS (817)	[27]
Wiedswang	CKs (AE1/AE3)	14	DFS, BCSS, DDFS (341)	[66]

\*Confirmed by multivariate analysis.

BCSS: Breast cancer-specific survival; CK: Cytokeratin; DDFS: Distant-DFS; DFS: Disease-free survival; EMA: Epithelial membrane antigen; OS: Overall survival.

A negative BM finding may, therefore, represent an additional clinical marker to identify those node-negative patients who are cured by surgery alone and need no additional adjuvant chemotherapy [11,53]. According to present guidelines, more than 90% of node-negative patients are currently recommended for adjuvant systemic therapy, even though up to 70% of these patients are cured by loco-regional surgery alone [54]. In this context, it is noteworthy that several other studies found DTCs in BM even several years after surgery and adjuvant therapy, and it seems that the presence of DTCs after adjuvant treatment might be useful to identify patients with an increased risk for recurrence [55,56]. These results demonstrate that DTCs can reside in a latent state of dormancy for many years before they grow out into overt skeleton metastases.

#### Clinical relevance of CTCs in the blood

It would be advantageous if peripheral blood could be used as source for the clinically relevant detection of minimal residual disease, since it is easier to obtain than BM. This is of relevance especially in the context of repeated examinations in order to monitor treatment. However, the relevance of CTCs so far is much less clear than for DTCs in BM. Blood is only a transient compartment for tumor cells and it is possible that only a small fraction of CTCs survives and is subsequently capable of forming detectable metastases.

The clinical relevance of CTCs in patients with primary, nonmetastatic breast cancer is currently under investigation. Preliminary comparative

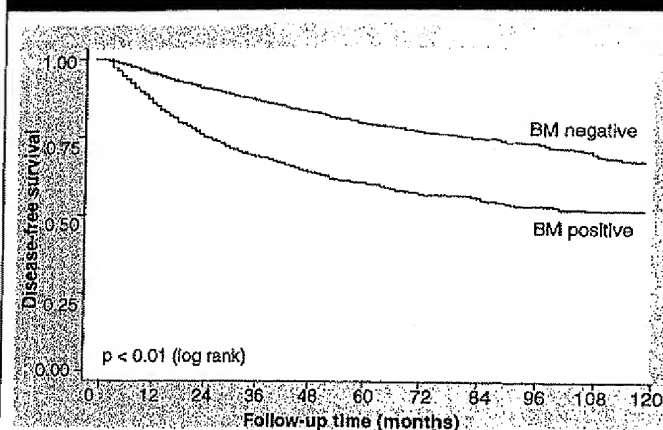
studies examining DTCs in BM and CTCs in these patients' blood found a correlation between the presence of tumor cells in both compartments [42,57], but the current findings do not support the replacement of BM analysis by blood testing [57,58]. However, it seems possible that blood analysis could deliver additional information and the monitoring of adjuvant therapy with repeated examinations deserves further attention.

In metastatic breast cancer, it was demonstrated that the detection of CTCs correlates with tumor progression and could, therefore, provide clinically relevant information [42,59]. In addition, by using an automated enrichment and analysis system, prognostic information was obtained [59,60]. Clinical studies must now show that the prognostic information derived from CTC detection can improve the outcome of patients, for example, by an earlier change of treatment.

#### Conclusion

The optimal method to detect DTCs would ensure that evidence for clinical relevance exists and that it is possible to perform these examinations in clinical routine. In view of this, the immunocytochemical approach with anti-CK antibodies appears to be the most suitable method for detecting DTCs currently available, owing to the specificity and the possibility of characterizing the cells morphologically. Many published studies indicate that BM is an important reservoir for DTCs and that the presence of these cells in BM indicates an increased metastatic capability of the primary tumor. However, studies on the prognostic

**Figure 3. Prognostic relevance of disseminated tumor cells in BM of breast cancer patients.**



Nodal-negative breast cancer patients who had DTCs in the BM (BM-positive) had a higher risk of cancer-related recurrence than patients with node-negative cancer who did not have DTCs in the BM (BM-negative). The median observation time of the survivors was 59 months (range, 12–120) and a total of 4703 primary breast cancer patients were included in this pooled analysis. BM: Bone marrow; DTC: Disseminated tumor cells. Reproduced with permission from [11].

relevance of DTCs in BM are sometimes difficult to interpret, as different groups use various antibodies to detect DTCs. Furthermore, inadequate numbers of patient cohorts with short follow-up times are reported in some studies. Nevertheless, there is significant evidence that the presence of DTCs in BM correlates with poorer prognosis. Moreover, the detection and characterization of DTCs in BM, as well as gene expression analyses related to early BM involvement, may lead to a

better understanding of the biology initiating metastatic spread in cancer patients and will eventually contribute to the development of more effective strategies to eliminate DTCs.

#### Future perspective

To date, no tool is available to assess whether a patient will individually profit from an adjuvant therapy after tumor removal, unless distant metastases arise, a situation that unavoidably leads to death. DTC should be an optimal target for new biological therapies (e.g., with antibodies) due to the low number of residual tumor cells. Monitoring the presence of DTCs in BM and/or CTCs in the peripheral blood during and after adjuvant therapy might help to monitor the response to systemic treatment in the future, and help to identify those patients who should receive an early change in therapy. Repeated BM sampling to examine DTCs cannot be easily realized in common clinical study settings for solid tumor patients, although it is a routine method for the staging and monitoring of leukemia patients. Serial examination of blood for CTCs might therefore be more acceptable for patients and clinical investigators, although the clinical data still need to prove the relevance of these cells in early-stage patients without overt metastases.

The availability of a surrogate marker for monitoring the effectiveness of a treatment would be of great value for the evaluation and development of new adjuvant therapies. A promising approach to achieve this goal might be the periodic screening of BM and peripheral blood during therapy for the presence of DTCs or CTCs.

#### Executive summary

##### Detection of disseminated tumor cells

- Disseminated tumor cells (DTCs) can be detected in the bone marrow (BM) of breast cancer patients at a frequency of 20–40%.
- The most important detection methods for DTC include immunocytochemical staining and molecular approaches (e.g., PCR).

##### Immunocytochemical staining

- Main limitation: time-consuming microscopical screening of slides and subjective read-out criteria.
- Immunocytochemical approaches with anticytokeratin antibodies are currently the most suitable method for detecting DTCs, owing to their specificity and also the possibility to characterize the DTCs morphologically in a normal light microscope.

##### PCR approach

- Main limitation: low-level transcription of the marker mRNA in normal cells (specificity problem).

##### Molecular & functional characterization of DTC

- Her2/neu-overexpressing DTCs in the BM of breast cancer patients characterize a clinically relevant subset of breast cancer micrometastases that is associated with poorer prognosis.
- Most DTCs and circulating tumor cells (CTCs) do not express the proliferation antigen Ki-67 and may therefore be relatively resistant to chemotherapy.
- Tumor cell dissemination appears to be a selective process predetermined by the phenotype of the primary tumor.

## Executive summary

**Clinical relevance of DTC detection in BM**

- The presence of DTC in BM predicts the occurrence of distant metastasis and is therefore associated with poorer survival.

**Clinical relevance of CTC in the blood**

- The detection of CTCs is a promising area of investigation and the clinical relevance of CTCs in patients with primary breast cancer is currently under investigation.
- Preliminary comparative studies examining DTCs in BM and CTCs in blood demonstrated a correlation between the presence of tumor cells in both compartments, but the current findings do not support the replacement of BM analysis by blood testing.

**Future directions**

- Single CTCs/DTCs in the blood and BM must be characterized for the identification of new targets for systemic adjuvant therapy aimed to eradicate micrometastatic disease.
- The detection of DTCs and/or CTCs in blood can be used to monitor adjuvant therapies (surrogate markers).

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